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EVALUATION OF COMMERCIAL-OFF-THE-SHELF MATERIALS FOR THE PRESERVATION OF GRAM-POSITIVE VEGETATIVE CELLS

Daniel Angelini
Jacquelyn Harris
Lisa Smith
Vipin Rastogi

RESEARCH AND TECHNOLOGY DIRECTORATE

Kristy Williams

EXCET, INC.
Springfield, VA 22151-2110

Laura Burton
Pooja Rastogi

OAK RIDGE INSTITUTE FOR SCIENCE AND EDUCATION
Oak Ridge, TN 37831-0117

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Angelini, Daniel; Harris, Jacquelyn; Smith, Lisa; Rastogi, Vipin (ECBC); Williams, Kristy (EXCET); Burton, Laura; and Rastogi, Pooja (ORISE)

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Director, ECBC, ATTN: RDCB-DRB-D, APG, MD 21010-5424
Excet, Inc.; 8001 Braddock Rd., Suite 303, Springfield, VA 22151-2110
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There is a pressing need to preserve the viability of biological threat agents collected in the field. Despite major gains in nucleic acid sequencing technologies, true positive identification of threat agents must be made through direct culture of the agents themselves. Materials have been developed to preserve the viability of pathogens contained within clinical samples, but many of these have not been examined for the ability to preserve biological materials relevant to the U.S. military (e.g. *Bacillus anthracis*). The purpose of this study was to account for possible delays from sample collection to laboratory analysis that often occur in the field for military-relevant, Gram-positive vegetative cells. The current study evaluated the effectiveness of 17 different materials capable of allowing nonrefrigerated storage of a sporulation-deficient strain of *B. anthracis* Sterne. The materials were evaluated for preservation effectiveness over a 14 day period at three different temperatures (4, 22, and 40 °C). Polymerase chain reaction analyses were performed on samples that did not show any viability at the end of the study to determine if nucleic acids were preserved. The study also evaluated the sampling efficiency and decay rates of *B. anthracis* vegetative cells that were spotted on two different surfaces.

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PREFACE

The work described in this report was authorized by the Defense Threat Reduction Agency (DTRA). The work was started in October 2015 and completed in October 2016.

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This report has been approved for public release.

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1. INTRODUCTION

Effective biological sampling from environmental surfaces and complex structures is essential to virtually every level of bio-release incident response, including sample collection, investigation, and analysis (Edmonds, 2009; Piepel et al., 2012). In many cases, collected biological samples cannot be analyzed in a timely manner, and therefore, must be preserved or stabilized to minimize decay and optimize viability and fidelity. Typically, this is accomplished through refrigeration or other types of cold storage; however, these types of preservation processes are resource intensive or virtually impossible in austere or denied areas. This is a technical capability gap that needs to be addressed because culturing a sample to determine what microorganisms are present remains essential, despite advances in genomics and proteomics. For example, culturing and growing a biothreat microorganism remains the "gold standard" for identification in a Sample Receipt Facility (Sanderson et al., 2004). Identification of biothreats using this method ensures that the samples were collected from an active culture and were not the result of residual environmental contamination.

Major threat agents such as *Bacillus anthracis*, *Yersinia pestis*, and *Burkholderia mallei* are endemic in many parts of the world; therefore, a positive polymerase chain reaction (PCR) may not indicate a deliberate biological attack. The results from a recent study that sequenced biological samples collected from the New York City subway system suggested the presence of *Y. pestis* and *B. anthracis*, even though no deliberate contamination was verified (Afshinnekoo et al., 2015). In fact, as of 2015, there have been no reported cases of naturally occurring *Y. pestis* infection within 1000 miles of New York City (Ackelsberg et al., 2015). These examples suggest that for threat assessment, it is essential to culture these agents in the laboratory to enable fully informed decisions. These results are expected to provide decision makers with the most accurate information about the presence of an active biological agent and allow for appropriate operational responses.

The main objective of this study was to enable sample acquisition to be separated from analysis without the logistical burdens that are associated with refrigeration or rapid transportation for vegetative cells. This study evaluated the effectiveness of 17 different potential solutions that are capable of allowing for nonrefrigerated storage of a sporulation-deficient strain of *B. anthracis* Sterne. The solutions were evaluated for effectiveness over a period of 14 days at three different temperatures (4, 22, and 40 °C). Real-time PCR analyses were performed on samples that did not show any viability at the end of the study. In additional experiments, the study evaluated the sampling efficiency and decay rates of the *B. anthracis* Sterne vegetative cells on two different surfaces (stainless steel and painted concrete).

2. MATERIALS AND METHODS

2.1 Reagents, Mediums, and Biological Preservatives

All chemicals and bacterial mediums were molecular grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). Butterfield's phosphate buffer was prepared in accordance with the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (FDA, 2013). In this study, 17 different potential biological preservative solutions were evaluated. Table 1 lists the selected preservation systems and the associated manufacturers.

Table 1. Preservation Materials

No.	Preservation System	Manufacturer	Catalog Number
1	Butterfield's phosphate buffer	FDA formulation	–
2	All-In-One swab kit	QuickSilver Analytics, Inc. (Belcamp, MD)	10193
3	Biomatrica custom formulation no. 1	Biomatrica, Inc. (San Diego, CA)	–
4	Biomatrica custom formulation no. 2	Biomatrica, Inc.	–
5	BBL CultureSwab	Becton, Dickinson, and Company (Franklin Lakes, NJ)	220099
6	BD ESwab	Becton, Dickinson, and Company	220245
7	Buffered peptone water (BPW), 5 g/mL	Sigma-Aldrich Co., LLC	77187
8	BPW, 20 g/mL	Sigma-Aldrich Co, LLC	77187
9	Copan ESwab	Copan Diagnostics, Inc. (Murrieta, CA)	480C
10	Copan swab-rinse kit (SRK)	Copan Diagnostics, Inc.	R4160
11	Puritan liquid Amies transport kit	Puritan Medical Products Company (Guilford, ME)	LA-116
12	Remel Sanicult transport swabs	Thermo Fisher Scientific, Inc.	R723140
13	Skim milk (filtered)	Cloverland Farms Dairy (Baltimore, MD)	–
14	Spent tryptic soy broth (TSB)	Thermo Fisher Scientific, Inc.	R112731
15	TSB, diluted 1/50	Thermo Fisher Scientific, Inc.	R112731
16	TSB, diluted 1/100	Thermo Fisher Scientific, Inc.	R112731
17	TSB, diluted 1/1000	Thermo Fisher Scientific, Inc.	R112731

–, not applicable.

2.2 Biological Agent Preparation

A sporulation-deficient strain of *B. anthracis* Sterne (*B. anthracis spo*$^{-}$) was obtained from Dr. Stephen Leppla at the National Institutes of Health (Bethesda, MD) (Sastalla et al., 2010). Glycerol stocks of *B. anthracis spo*$^{-}$ were streaked on tryptic soy agar (TSA) plates,

and colonies appeared after the plates were incubated at 37 °C for 24 h. A single colony was selected from the plate and individually inoculated into four separate 50 mL conical tubes containing 10 mL of sterile TSB. These tubes were then placed in a laboratory shaker at 37 °C and processed at 200 rpm for 24 h. Following this growth step, the cultures were centrifuged, the supernatant was removed, and then the cultures were washed three times with 10 mL of sterile Butterfield's buffer. After the final wash, 50 µL of the washed cultures were added to 950 µL of Butterfield's buffer in 1.5 mL conical tubes. These samples were then centrifuged and resuspended in 1 mL of the experimental preservative solution.

2.3 Viability Testing

The prepared *B. anthracis spo*$^-$ vegetative cells were stored at 4, 22, or 40 °C for up to 14 days in the selected preservatives. At the end of each time point (0, 1, 3, 4, 7, and 14 days), 100 µL of suspension was removed and serially diluted in Butterfield's buffer. These dilutions were then streaked on TSA plates in duplicate. Day 0 samples were taken immediately after preparation of the cells. The plates were placed at 37 °C for 16–24 h, and then the resultant colonies were evaluated using a QCount colony counter (Advanced Instruments, Inc.; Norwood, MA) to determine the bacterial load. Results are expressed as the Log_{10} of recovered colony-forming units (CFU/mL) or the percentage of viability as compared to Day 0 (Rastogi et al., 2010; Hubbard et al., 2011).

2.4 Real-Time PCR

Real-Time PCR was performed on samples that produced no viable cells at the end of the 14 day experimental test period using the 96-well FastBlock format on the Applied Biosystems 7900HT instrument (Thermo Fisher Scientific, Inc.). The *B. anthracis* Target 3 FastBlock MasterMix, Catalog no. PCR-BAT-3FB-K, obtained from the Defense Biological Product Assurance Office (DBPAO; Frederick, MD), was used for PCR analysis. Experimental samples consisted of 5 µL of nonviable cell suspension. To prepare the samples, 5 µL of sample (negative or positive control or experimental sample) was added to 15 µL of DBPAO MasterMix and pipetted into the wells of an Applied Biosystems 7900HT Fast Thermal Cycling plate. The samples were run in two stages on the PCR instrument. Stage one consisted of one cycle at 50 °C for 2 min and one cycle at 95 °C for 20 s. Stage two consisted of 45 cycles at 95 °C for 1 s followed by 60 °C for 20 s (Betters et al., 2014; Buttner et al., 2004). Targets were considered positive if the cycle threshold (Ct) was <40. For statistical analysis in this study, undetermined samples were given a Ct of 40.

2.5 Decay and Sampling Study

In these studies, single colonies of *B. anthracis spo*$^-$ cells were selected from a previously streaked TSA plate and individually inoculated into 50 mL conical tubes containing 10 mL of sterile TSB. These tubes were then placed in a laboratory shaker at 37 °C at 200 rpm for 24 h. At the end of this incubation, 1 mL of inoculum was spotted on 2 × 2 in. stainless steel or 2 × 2 in. painted concrete surfaces and allowed to dry for approximately 3 h (R.G. Collins Glass Company; Dundalk, MD). The paint used for the concrete surfaces was Glidden White Interior 1456 Latex Semi-Gloss (PPG Industries, Pittsburgh, PA). Once the surfaces were dry,

we immediately performed surface sampling using the swab provided with the Remel Sanicult transport kit, and additional samples were taken at a 1 h post-drying point. These samples were collected according to a surface-sampling method for *B. anthracis* that was developed for First Responders by the U.S. Centers for Disease Control and Prevention (CDC, 2012). The swab was initially used in a horizontal overlapping "S" pattern, followed by a vertical "S" pattern, and finally in a diagonal "S" pattern. The end of the swab was cut off using sterile scissors and placed in a 50 mL conical tube filled with 10 mL of Butterfield's buffer plus 0.01% Tween 80. The filled tube was then vortexed for two min to aid in the removal of the cells from the collection device. Finally, the samples were plated, and the viability was assessed, as described in Section 2.3, Viability Testing.

3. RESULTS

3.1 Enumeration of *B. anthracis spo*⁻ Vegetative Cells

Before the start of the extended storage studies, titers of *B. anthracis spo*⁻ were evaluated for consistent growth in TSB. Four experiments were performed; each consisted of three individual replicates of *B. anthracis spo*⁻ cells inoculated in 10 mL of TSB. These cells were then grown at 37 °C for 24 h in an upright shaking incubator set at 200 rpm. Following this, the cultures were titered to determine the amount of CFU/mL in each (Figure 1). The results consistently showed approximately 10^8 CFU/mL in each of the 24 h 10 mL cultures. These procedures were then used for the cultures that were needed for the extended storage studies.

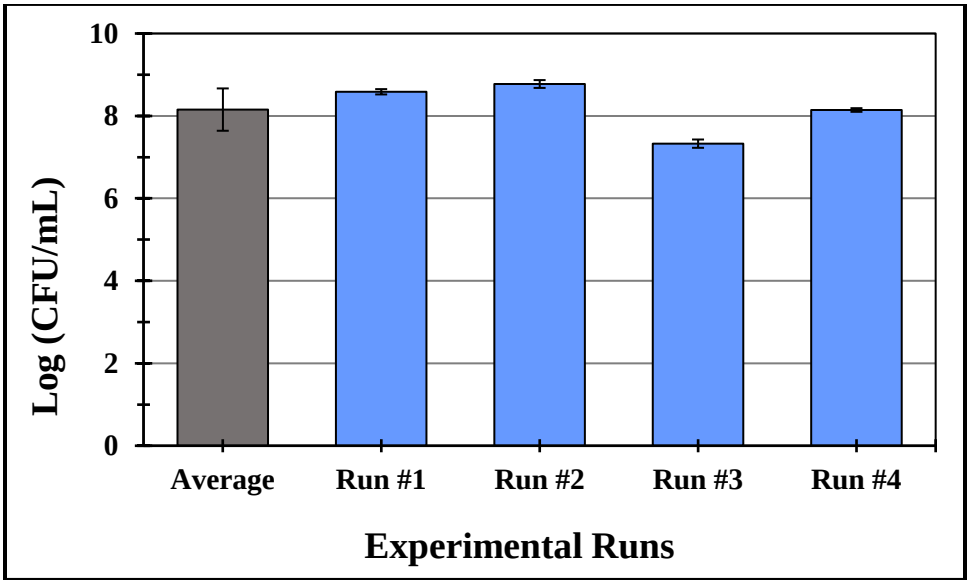

Figure 1. Titer for *B. anthracis spo*⁻ grown in TSB overnight. Results are expressed as the mean ± standard deviation (SD) of the calculated log (CFU/mL) from each culture. The number of replicates (*n*) for each run is equal to 3.

3.2 Effect of Extended Storage on *B. anthracis spo*⁻ Vegetative Cells at 4 °C

Seventeen different potential preservation systems were evaluated for storage of *B. anthracis spo*⁻ vegetative cells at 4 °C for up to 14 days. These results are displayed in Table 2. At the end of a 14 day storage period, 9 of the 17 preservation systems retained viable *B.anthracis spo*⁻ vegetative cells. Only the Remel Sanicult transport swabs system retained almost all of the initial viability as seen on Day 0. Of the other 9 materials that retained viability, 7 of these retained at least 1 log of viable cells.

Table 2. Bio-Preservation of *B. anthracis spo*⁻ at 4 °C

Storage Temperature	Preservation System	Log (CFU/mL) ± SD				
		Day 0	Day 1	Day 3	Day 7	Day 14
4 °C	Butterfield's phosphate buffer	5.45 ± 0.55	3.94 ± 1.73	2.92 ± 2.22	1.97 ± 1.50	–
	All-in-One swab kit	6.01 ± 0.07	2.20 ± 2.05	1.57 ± 1.43	–	–
	BBL CultureSwab	5.44 ± 0.29	5.33 ± 0.32	5.10 ± 0.36	4.71 ± 0.55	2.87 ± 1.31
	BD Eswab	6.03 ± 0.67	5.65 ± 0.33	4.99 ± 1.24	1.62 ± 1.78	-
	Biomatrica custom formulation no. 1	4.79 ± 0.33	3.89 ± 0.29	2.85 ± 0.39	3.07 ± 0.36	1.10 ± 1.21
	Biomatrica custom formulation no. 2	4.96 ± 0.38	4.22 ± 0.15	2.92 ± 0.68	3.31 ± 0.25	2.24 ± 0.30
	BPW, 5 g/L	6.04 ± 0.09	5.96 ± 0.15	5.55 ± 0.39	1.46 ± 2.26	1.09 ± 0.92
	BPW, 20 g/L	6.03 ± 0.09	6.01 ± 0.16	5.60 ± 0.42	3.49 ± 1.73	1.58 ± 1.41
	Copan Eswab	6.06 ± 0.64	5.66 ± 0.32	5.51 ± 0.63	2.50 ± 2.74	–
	Copan SRK	6.06 ± 0.24	6.08 ± 0.12	0.45 ± 1.10	3.07 ± 0.66	–
	Puritan liquid Amies transport kit	5.97 ± 0.77	5.80 ± 0.90	5.09 ± 1.20	2.25 ± 2.47	–
	Remel Sanicult transport swabs	5.84 ± 0.66	5.63 ± 0.56	5.70 ± 0.71	5.52 ± 0.74	5.60 ± 0.71
	Skim milk (filtered)	6.37 ± 0.15	5.17 ± 0.74	4.05 ± 1.13	2.97 ± 0.54	0.96 ± 0.81
	Spent TSB	6.18 ± 0.18	6.08 ± 0.17	5.82 ± 0.25	5.28 ± 0.40	2.12 ± 0.30
	TSB, diluted 1/50	6.06 ± 0.12	5.42 ± 0.21	4.47 ± 0.56	3.25 ± 1.92	1.30 ± 1.43
	TSB, diluted 1/100	6.10 ± 0.09	4.87 ± 0.25	3.93 ± 0.89	2.72 ± 1.54	0.75 ± 0.84
	TSB, diluted 1/1000	6.12 ± 0.05	3.12 ± 1.56	1.85 ± 1.38	0.17 ± 0.41	–

Notes: Results are expressed as the mean ± SD of the reported recovered log (CFU/mL). Experimental $n \geq 6$ for each condition. –, not applicable.

3.3 Effect of Extended Storage on *B. anthracis spo*⁻ Vegetative Cells at 22 °C

Preservation systems were also examined for effectiveness at 22 °C (Table 3). After 14 days of storage, all of the 17 materials tested retained at least 2 logs of viable cells.

Table 3. Bio-Preservation of *B. anthracis spo*⁻ at 22 °C

Storage Temperature	Preservation System	Log (CFU/mL) ± SD				
		Day 0	Day 1	Day 3	Day 7	Day 14
22 °C	Butterfield's phosphate buffer	5.45 ± 0.50	5.20 ± 0.33	5.18 ± 0.22	4.41 ± 0.40	2.95 ± 0.50
	All-in-One swab kit	6.01 ± 0.08	5.32 ± 0.38	4.76 ± 0.37	4.81 ± 0.05	4.25 ± 0.14
	BBL CultureSwab	5.23 ± 0.51	5.55 ± 0.41	4.73 ± 1.19	4.95 ± 0.63	4.91 ± 0.50
	BD Eswab	6.12 ± 0.53	5.98 ± 0.70	5.53 ± 0.51	5.37 ± 1.31	5.15 ± 0.38
	Biomatrica custom formulation no. 1	4.50 ± 0.13	5.02 ± 0.35	5.22 ± 0.51	4.93 ± 0.20	3.64 ± 0.56
	Biomatrica custom formulation no. 2	5.11 ± 0.47	4.77 ± 0.10	5.59 ± 0.17	4.49 ± 1.11	4.04 ± 0.51
	BPW, 5 g/L	6.05 ± 0.04	6.19 ± 0.18	6.04 ± 0.12	5.96 ± 0.14	6.04 ± 0.23
	BPW, 20 g/L	6.06 ± 0.03	6.33 ± 0.23	6.21 ± 0.14	5.49 ± 0.40	5.82 ± 0.30
	Copan Eswab	5.97 ± 0.69	5.71 ± 0.88	5.06 ± 0.95	5.53 ± 1.16	5.28 ± 0.24
	Copan SRK	6.36 ± 0.43	6.19 ± 0.43	3.10 ± 1.00	2.80 ± 0.72	3.02 ± 0.46
	Puritan liquid Amies transport kit	6.38 ± 0.43	6.26 ± 0.45	5.53 ± 0.48	5.51 ± 1.23	5.68 ± 0.22
	Remel Sanicult transport swabs	5.89 ± 0.75	5.95 ± 0.63	6.06 ± 0.48	5.54 ± 0.20	3.74 ± 2.81
	Skim milk (filtered)	6.38 ± 0.18	6.90 ± 0.14	6.90 ± 0.15	5.89 ± 0.14	5.80 ± 0.15
	Spent TSB	6.14 ± 0.17	6.35 ± 0.20	6.69 ± 0.11	6.48 ± 0.46	6.57 ± 0.20
	TSB, diluted 1/50	6.03 ± 0.08	6.23 ± 0.11	5.85 ± 0.12	5.53 ± 0.08	4.91 ± 0.17
	TSB, diluted 1/100	6.12 ± 0.08	6.06 ± 0.48	5.70 ± 0.39	5.70 ± 0.14	3.49 ± 1.15
	TSB, diluted 1/1000	6.03 ± 0.11	5.64 ± 0.17	5.36 ± 0.22	4.02 ± 0.09	2.45 ± 0.69

Notes: Results are expressed as the mean ± SD of the reported recovered log (CFU/mL). Experimental $n \geq 6$ for each condition.

3.4 Effect of Extended Storage on *B. anthracis spo*⁻ Vegetative Cells at 40 °C

In the final set of preservation studies, 17 different potential preservation systems were evaluated for storage of *B. anthracis spo*⁻ vegetative cells at 40 °C for up to 14 days. These results are shown in Table 4. At the end of a 14 day storage period, 11 of the 17 preservation systems retained at least 1 log of viable *B. anthracis spo*⁻ vegetative cells. The Remel Sanicult transport swabs, BPW at 5 g/L, and BPW at 20 g/L retained at least 5 logs of viable *B. anthracis spo*⁻ cells. The filtered skim milk and spent TSB retained at least 4 logs and 3 logs of viable cells, respectively. The other six systems examined retained at least 1 log of viable *spo*⁻ cells.

Table 4. Bio-Preservation of *B. anthracis spo*⁻ at 40 °C

Storage Temperature	Preservation System	Log (CFU/mL) ± SD				
		Day 0	Day 1	Day 3	Day 7	Day 14
40 °C	Butterfield's phosphate buffer	5.40 ± 0.48	2.29 ± 2.26	2.00 ± 2.40	0.39 ± 1.16	1.09 ± 1.65
	All-in-One swab kit	6.05 ± 0.08	4.41 ± 0.45	3.81 ± 0.24	2.98 ± 1.01	–
	BBL CultureSwab	5.09 ± 0.33	3.03 ± 2.53	1.83 ± 2.02	1.21 ± 1.88	–
	BD Eswab	6.16 ± 0.70	2.52 ± 2.79	1.09 ± 1.69	–	–
	Biomatrica custom formulation no. 1	4.38 ± 0.44	4.97 ± 0.43	2.33 ± 1.97	–	–
	Biomatrica custom formulation no. 2	5.13 ± 0.19	4.53 ± 0.17	4.19 ± 0.12	–	–
	BPW, 5 g/L	6.06 ± 0.04	5.13 ± 0.30	5.66 ± 0.36	5.47 ± 0.28	5.19 ± 0.54
	BPW, 20 g/L	6.07 ± 0.02	5.41 ± 0.46	5.81 ± 0.42	5.70 ± 0.52	5.88 ± 0.50
	Copan ESwab	6.07 ± 0.59	2.52 ± 2.79	1.42 ± 1.51	–	1.31 ± 2.18
	Copan SRK	6.09 ± 0.14	–	–	–	–
	Puritan liquid Amies transport kit	6.19 ± 0.47	2.60 ± 2.85	2.81 ± 3.08	2.36 ± 2.59	1.82 ± 2.82
	Remel Sanicult transport swabs	5.89 ± 0.57	5.52 ± 0.20	5.64 ± 0.25	5.68 ± 0.23	5.59 ± 0.37
	Skim milk (filtered)	6.48 ± 0.18	6.19 ± 0.12	5.58 ± 0.13	4.88 ± 0.42	5.05 ± 0.48
	Spent TSB	6.28 ± 0.20	5.85 ± 0.19	3.89 ± 3.03	3.01 ± 2.89	3.01 ± 2.89
	TSB, diluted 1/50	6.15 ± 0.08	3.84 ± 2.11	5.44 ± 0.66	4.30 ± 0.91	1.76 ± 1.95
	TSB, diluted 1/100	6.14 ± 0.03	4.61 ± 0.74	2.89 ± 2.39	2.31 ± 2.62	1.45 ± 1.72
	TSB, diluted 1/1000	6.09 ± 0.05	1.17 ± 1.82	2.95 ± 2.31	3.07 ± 1.77	2.36 ± 1.85

Notes: Results are expressed as the mean ± SD of the reported recovered log (CFU/mL). Experimental $n \geq 6$ for each condition. –, not applicable.

3.5 Enhanced Cell Preservation Using the Five Best-Performing Materials

Figure 2 shows graphical representations of the five best-performing cellular preservatives in this study (Remel Sanicult transport swabs; BPW, 5 g/L; BPW, 20 g/L; skim milk [filtered]; and spent TSB), as compared with the reference buffer (Butterfield's Buffer).

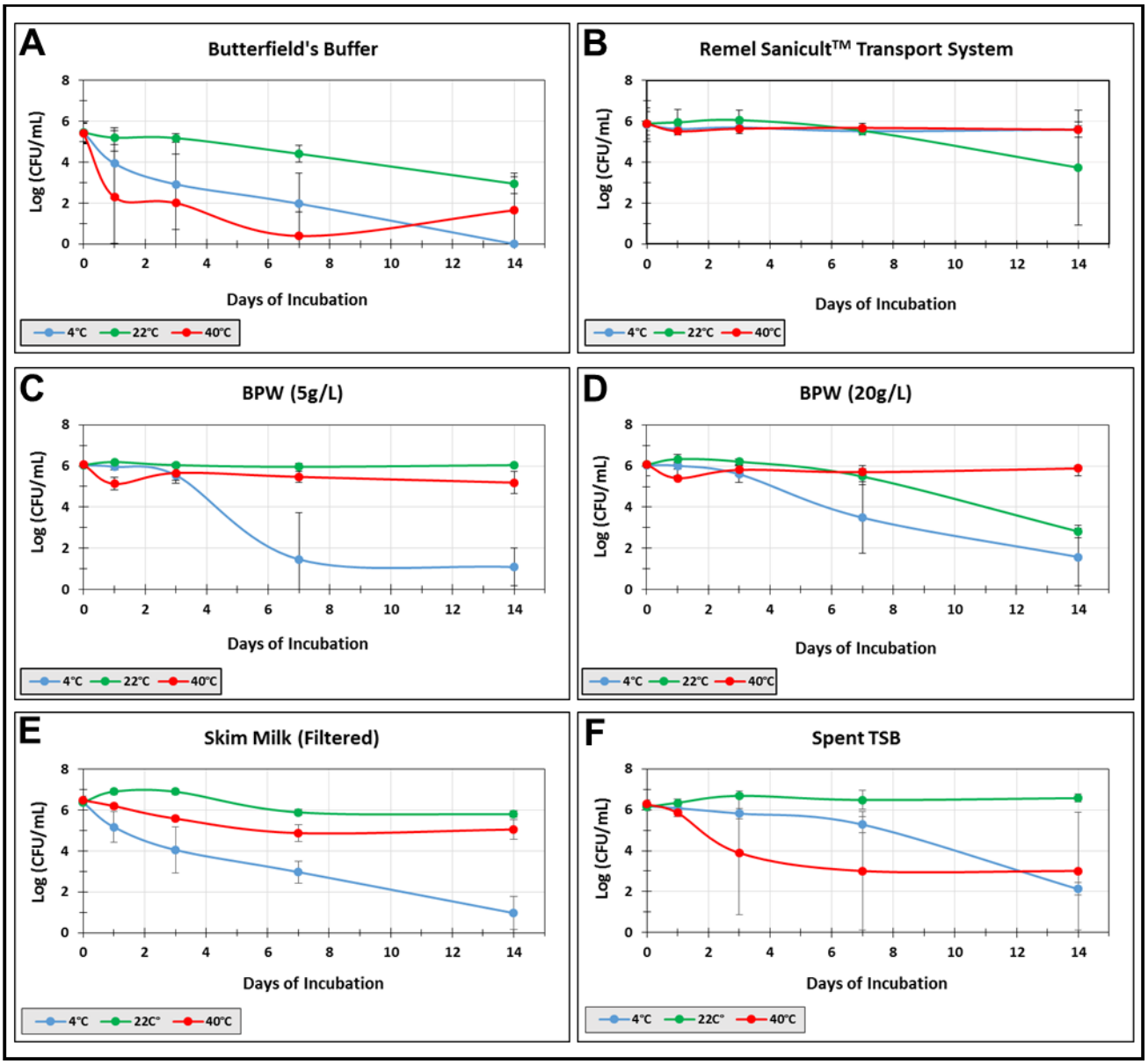

Figure 2. Sustained cell preservation using the five best-performing materials. Results are expressed as the mean ± SD of the calculated log (CFU/mL) from each culture. (A) Butterfield's Buffer; (B) Remel Sanicult transport swabs; (C) BPW, 5 g/L; (D) BPW, 20 g/L; (E) skim milk (filtered); and (F) spent TSB. Blue lines represent storage at 4 °C. Green lines represent storage at 22 °C. Red lines represent storage at 40 °C. The number of replicates (*n*) for each run was ≥6.

3.6 Effect of Extended Storage on PCR Results

Experimental samples that retained no detectable viable *B. anthracis spo*⁻ cells were examined further to determine if the stored samples would still produce a positive PCR signal (Table 5). In these experiments, all of the samples displayed positive PCR results (except for the Biomatrica custom formulations nos. 1 and 2), with Ct values ranging from 18 to 22.

Table 5. Genomic DNA Assessment by PCR Amplification

Preservation System	Temperature (°C)	Ct
All-in-One swab kit	4	18.83 ± 0.07
All-in-One swab kit	40	18.65 ± 0.24
Butterfield's phosphate buffer	4	19.31 ± 0.21
Butterfield's phosphate buffer	40	18.43 ± 0.23
BBL CultureSwab	40	20.71 ± 0.32
BD Eswab	4	21.71 ± 2.63
BD Eswab	40	21.79 ± 2.60
Biomatrica custom formula no. 1	40	UD
Biomatrica custom formula no. 2	40	UD
Puritan liquid Amies transport kit	4	20.65 ± 0.17
TSB, diluted 1/1000	4	21.05 ± 0.48

Notes: PCR was performed as described in Section 2.4, Real-Time PCR. Results are displayed as the mean ± SD of the reported Ct values. UD, undetermined. Experimental $n \geq 3$ for each condition.

3.7 Decay and Sampling Study Results

In these studies, approximately 8.5 log of *B. anthracis spo*$^-$ (in 1 mL of growth media) was spotted on either a 2 × 2 in. stainless steel or painted concrete surface and allowed to dry for approximately 1–3 h to determine if sampling affected the preservation capability of the Remel Sanicult transport kit. Samples were collected from each surface using the swabs provided with the Remel Sanicult transport kit, as soon as the surface was dry and 1 h after completion of drying. The collected samples were then processed as stated in Section 2.5, Decay and Sampling Study. Results of this study indicated that most of the *B. anthracis spo*$^-$ cells become nonviable after the initial drying phase (Figure 3). These results show that <1 log of the initial cells remained on either of the two surfaces examined in this study.

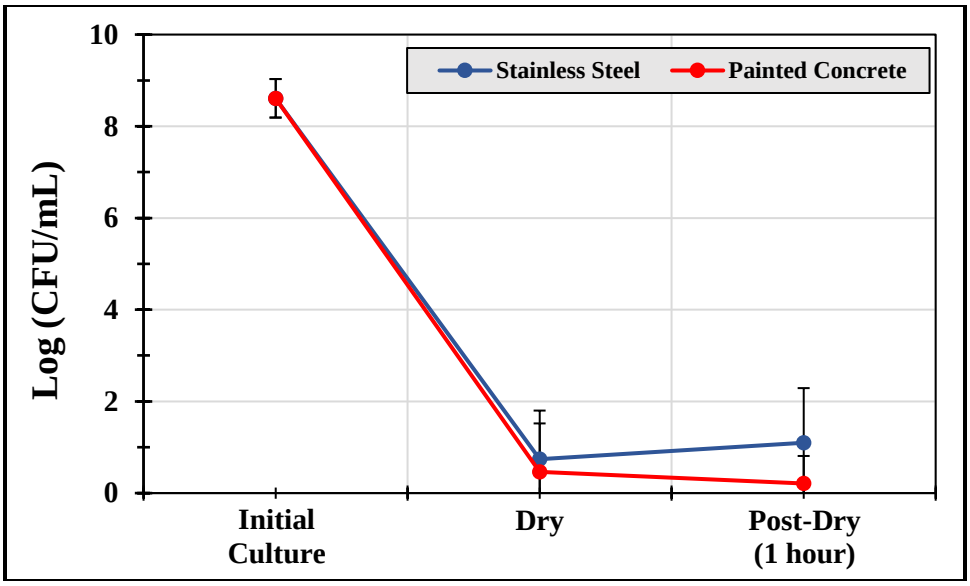

Figure 3. *B. anthracis spo*⁻ sampling time course. Results are expressed as the mean ± SD of the log (CFU/mL) recovered. The experimental $n = 8$ for each condition.

4. CONCLUSIONS

This study evaluated 17 different solutions (Table 1) that had the potential to preserve the viability of *B. anthracis spo*⁻ vegetative cells for up to 14 days under three different temperature conditions (4, 22, and 40 °C). All of the solutions examined in this study were capable of preserving viability at the 22 °C temperature (Table 3); however, preserving viability at 4 and 40 °C proved to be more difficult (Tables 2 and 4).

The following results were obtained with storage at 4 °C:

- Day 3: Viable cells were detected in all of the solutions.
- Day 7: No viable cells were detected in the All-in-One swab kit.
- Day 14: Viable cells (Table 2) were detected in 10 of the 17 solutions.

Only the Remel Sanicult transport swabs solution maintained the level of viability that was seen at Day 0 of the experiment. The Biomatrica custom formulation no. 2, the BBL CultureSwab solution, and the Spent TSB retained at least 2 logs of viable cells from the initial cell count. The remaining materials: Biomatrica custom formulation no. 1, BPW dilutions, skim milk (filtered), and the TSB dilutions only retained approximately 1 log of the initial viable cells.

The 40 °C temperature (Table 4) was as challenging as the 4 °C condition in maintaining viable cells. The following results were obtained with storage at 40 °C:

- Day 1: No viable cells were detected in the Copan SRK solution.
- Day 3: Viable cells were detected in all of the solutions but Copan SRK.

- Day 7: No viable cells were detected in the BD Eswab, Biomatrica custom formulations, and Copan ESwab solutions.
- Day 14: No viable cells were detected in the All-in-One swab kit and the BBL CultureSwab solutions. Viable cells were detected in the Remel Sanicul transport swabs solution and both dilutions of BPW at a level that was similar at the end of the 14 day study as compared to the starting concentrations of viable *B. anthracis spo*$^{-}$ cells. The skim milk (filtered) and the spent TSB preservation systems retained 4.75 logs and 2.8 logs of viable cells, respectively.

Overall, these five preservation systems—Remel Sanicul transport swabs, BPW 5 and 20 g/L, skim milk (filtered), and spent TSB—provided the best results and retained viable cells for 14 days under a variety of temperature conditions.

The systems that sustained no detectable viable cells at the end of the 14 day study were evaluated through PCR to determine if there were any intact nucleic acids following storage (Table 5). These solutions included the All-in-One swab kit (4 and 40 °C), Butterfield's phosphate buffer (4 and 40 °C), the BBL CultureSwab (40 °C), BD ESwab (4 and 40 °C), the Biomatrica custom formulations nos. 1 and 2 (40 °C), the Puritan liquid Amies (4 °C), and the 1/1000 dilution of TSB (4 °C). Analysis of all of these solutions, except the Biomatrica custom formulations nos. 1 and 2 (40 °C), resulted in positive PCR results. The Ct values for these samples ranged from 18 to 22.

Additional studies were performed to determine whether sampling from different surfaces affected the ability of the Remel Sanicult transport swab system to preserve *B. anthracis spo*$^{-}$ cells (Figure 3). The results indicated that most of these cells (>7 logs) lost viability after drying. The data indicated that *B. anthracis spo*$^{-}$ cells require moisture to stay viable for any length of time.

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ACRONYMS AND ABBREVIATIONS

BPW	buffered peptone water
CDC	U.S. Centers for Disease Control and Prevention
CFU	colony-forming units
Ct	cycle threshold
DBPAO	Defense Biological Product Assurance Office
FDA	U.S. Food and Drug Administration
PCR	polymerase chain reaction
SD	standard deviation
SRK	swab-rinse kit
TSA	tryptic soy agar
TSB	tryptic soy broth

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RDCB-DRB-D
ATTN: Angelini, D.
Harris, J.
Rastogi, V.
Rosenzweig, N.

Defense Threat Reduction Agency
J9-CBS
ATTN: Graziano, A.

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